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**14. ABSTRACT**

In the C3(1)/SV40 T-antigen (Tag) FVB/N mouse model of human estrogen and progesterone receptor-negative breast cancer, the stress response elicited by social isolation is associated with increased expression of metabolic genes in the mammary gland. To further understand accelerated tumor growth associated with social isolation, we separated mammary gland adipocytes from ductal epithelium and stroma and then analyzed individual fractions for changes in metabolic gene expression and function. The increased expression of the key metabolic genes Acaca, Hk2 and Acly was found to be significantly elevated in the adipocytes of the mammary gland, and surprisingly, was not significantly increased in visceral adipose depots of socially isolated female mice. Increased metabolic gene expression in the mammary gland of socially isolated mice coincided with increased glucose metabolism, lipid synthesis, and leptin expression. Furthermore, culture media from isolated versus group-housed mouse mammary adipose tissue resulted in relatively increased proliferation of mammary cancer cells. These results suggest that exposure to chronic social isolation results in metabolic changes in mammary gland adipocytes that contribute to increased growth of adjacent epithelial cell tumors. We propose a model in which environmental stress affects estrogen-independent mammary tumor growth, at least in part, through changes in mammary adipocyte biology.

**15. SUBJECT TERMS**
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## Introduction

Using a whole genome approach, we previously identified key lipid synthesis genes including acetyl-CoA carboxylase alpha *(Acaca)*, Hexokinase 2 *(Hk2)*, and ATP citrate lyase *(Acly)* as significantly over-expressed in mammary gland tissue from isolated compared to group-housed SV40 Tag mice. In our earlier study, RNA was obtained from whole mammary gland tissue so we were unable to determine the cell type(s) contributing to the increase in metabolic gene expression. Because it has become increasingly clear that mammary epithelial cell proliferation is influenced by the adjacent non-epithelial cells (16), we sought to establish the specific cell types contributing to changes in whole mammary gland gene expression. Our current findings reveal an association between social isolation and mammary gland adipose tissue metabolism, without a measurable concomitant effect on systemic metabolism. These results implicate mammary adipocyte function as an important modulator of estrogen receptor-independent breast cancer growth.

**Body**

Human epidemiological studies have revealed that social isolation is associated with an increased risk of both all-cause mortality and metabolic diseases such as diabetes (1). Although association studies examining social isolation and human cancer risk have had mixed results (2)-4), the conclusions of these studies are likely inconsistent because of the genetic and environmental variation inherent in human populations, as well as the extensive heterogeneity of breast cancer subtypes (3, 4). These issues make identifying underlying mechanisms connecting social stressors to breast cancer biology challenging. They also underscore the importance of developing well-defined preclinical models for identifying the variety of connections between social stressors and specific cancer subtypes.

Recent animal models of breast cancer have examined imposed social isolation, a well-defined chronic stressor for female rodents, and found an association with increased mammary tumor growth and malignancy. For example, in SV40 T-antigen (Tag) FVB/N mice (5) and Sprague-Dawley rats (6), social isolation is associated with larger mammary gland tumor burden and increased tumor invasiveness independently of circulating estrogen and progesterone levels. Furthermore, our laboratories discovered that several genes encoding key enzymes regulating lipid metabolism are differentially regulated in the mammary glands of chronically socially isolated versus group-housed mice, even prior to invasive tumor development (7).

Interestingly, the isolated mice go on to develop larger invasive mammary gland tumors compared to group-housed mice, raising hypothesis that changes in lipid metabolism (either in the pre-malignant epithelial cells or the surrounding adipocytes and stromal cells) may be driving the more aggressive mammary tumor growth of the social isolates. There is increasing evidence that mammary gland tumor development is influenced by both local and systemic metabolic factors (7, 8) in addition to local estrogenic factors that can promote ER+ breast cancer (7-9). Moreover, an emerging set of data links systemic metabolic diseases, such as the metabolic syndrome and central adiposity, to estrogen receptor negative (ER-) breast cancer risk in pre-menopausal women (10, 11).

In mouse models, social stressors have been previously linked to obesity (12), disruption of lipid metabolism (13, 14), and the development of type 2 diabetes (15), thereby supporting an association between exposure to social stressors and the development of metabolic diseases. However, mechanisms through which disrupted peripheral metabolic cues promote mammary tumorigenesis are still unclear. Adding further complexity, the stromal compartment, and its reciprocal communication with the mammary epithelium, is an important factor influencing breast cancer outcome (16). How the interactions between tumor and stroma are affected by local and peripheral metabolism remains a challenging puzzle.

In breast cancer, resident fibroblasts, hematopoietic, immune, and endothelial cells define a significant portion of the mammary stroma and have been the focus of many recent studies (16). However, mammary gland adipocytes are far less well understood components of the stroma. The relative lack of research into the role of the adipocyte microenvironment in breast cancer is surprising considering the abundance of adipose tissue in the mammary gland and the links between metabolism and breast cancer. This knowledge gap may be because adipose tissue was previously considered an inert energy storage tissue and has only recently been found to play a key endocrine role in mammalian physiology (17).

Endocrine secretion by adipocytes and adipose tissue includes the release of known growth factors, hormones, and cytokines as well as adipose-specific factors (adipokines), many of which have been implicated in cancer progression (18). For example, leptin, which normally functions to modulate satiety, may promote tumor progression through mitogenic signaling and promotion of angiogenesis (19). Adding complexity to the endocrine action of adipocytes are studies that have established that the adipose tissue location within an organism (e.g. visceral vs. subcutaneous) affects the type and relative amount of secreted factors and contributes to metabolic function, including insulin sensitivity (20). Those properties specific to mammary adipose tissue and the mammary microenvironment that could influence breast cancer biology remain largely unexplored. Therefore, it is not yet known whether abnormal functioning of mammary adipocytes and the ensuing effects on local metabolism contribute to breast cancer biology.

Using a whole genome approach, we previously identified key lipid synthesis genes including acetyl-CoA carboxylase alpha *(Acaca)*, Hexokinase 2 *(Hk2)*, and ATP citrate lyase *(Acly)* as significantly over-expressed in mammary gland tissue from isolated compared to group-housed SV40 Tag mice. In our earlier study, RNA was obtained from whole mammary gland tissue so we were unable to determine the cell type(s) contributing to the increase in metabolic gene expression. Because it has become increasingly clear that mammary epithelial cell proliferation is influenced by the adjacent non-epithelial cells (16), we sought to establish the specific cell types contributing to changes in whole mammary gland gene expression. Our current findings reveal an association between social isolation and mammary gland adipose tissue metabolism, without a measurable concomitant effect on systemic metabolism. These results implicate mammary adipocyte function as an important modulator of estrogen receptor-independent breast cancer growth.

**Social isolation versus group housing is associated with increased vigilance followed by accelerated mammary tumor growth**

Homozygous C3(1)/SV40 Tag FVB/N mice develop non-invasive mammary tumors beginning as early as 12 weeks of age and palpable tumors beginning at approximately 16 weeks (25). Our previous work demonstrated that socially isolated SV40 Tag mice have a larger invasive tumor burden compared to group-housed mice (7). These results needed to be reconfirmed in order to investigate the metabolic and molecular changes underlying the increased tumor burden.

Within this study, among all mice that had palpable tumors by 18 weeks of age (Fig 1A), isolated mice developed a significantly larger average tumor burden than group-housed mice (*$p=0.013$, Fig. 1B). We again observed that socially isolated mice became more vigilant by 16 weeks of age, regardless of newborn temperament, as demonstrated by a longer time to leave their home base and explore a novel environment ($p<0.0001$; log-rank test, Fig. 1C). These results confirm that exposure to social isolation is associated with increased mammary tumor growth and vigilance (7).

**Social isolation is associated with metabolic gene expression changes in mammary adipocytes**

Next, we sought to determine whether the increased tumor burden of socially isolated mice is associated with local and/or systemic changes in lipid metabolism. Our

previous studies in the SV40 Tag FVB/N model found that social isolation results in increased mRNA steady-state levels of key glycolytic and fat synthesis genes (*Hk2, Acaca*, and *Acly)* collected from whole mammary gland extracts from mice at 15 weeks of age, before palpable tumor formation.(7). We hypothesized that mammary gland adipocytes might account for the observed overall change in metabolic gene expression. Therefore, we used a collagenase digestion and centrifugation protocol (26) to separate the floating adipocytes from other cells in the mammary glands of 15-week old mice prior to palpable tumors. Representative images of fractionated cells are shown in Figure 2A. mRNA was isolated from each fraction and gene expression was analyzed by Q-RT-PCR.

A comparison of the relative gene expression in the adipocyte vs. stromal/epithelial fraction (regardless of housing condition) revealed 20-60 fold higher overall metabolic gene expression in the adipocyte (Fig. 2B). Adipocytes from socially isolated SV40 FVB/N mouse mammary glands expressed significantly higher steady-state *Acaca* ($2^{-\Delta\Delta Ct}$=2.94, ***p=0.0001), *Hk2* ($2^{-\Delta\Delta Ct}$=1.84, *p=0.012), and *Acly* ($2^{-\Delta\Delta Ct}$=2.93, ***p=0.0001) mRNA levels compared to mRNA from group-housed mammary gland adipocytes (Fig. 2C). In cells of the non-adipocyte fraction, expression of these genes was not statistically different between isolated and group-housed animals (Fig. 2D) ($p>0.12$ for all three genes, Fig 2C, Supplemental Table 1). These results indicate that metabolic gene expression changes in the mammary gland following social isolation occur primarily in the adipocyte fraction.

To determine whether the elevated metabolic gene expression was specific to the mammary gland depot or also present in other adipose tissue depots, we harvested visceral (gonadal) fat. Interestingly, there were no significant differences in the visceral fat metabolic gene expression from the isolated versus grouped mice (Fig. 2E, Supp. Table 1, $p>0.43$ for all three genes), suggesting that the upregulation in metabolic gene expression associated with social isolation is mammary fat depot-specific.

**Social isolation is not associated with detectable systemic metabolic changes**

Our gene expression data suggested the intriguing possibility that social isolation in rodents is associated with mammary adipose-specific metabolic changes; therefore, we performed additional analyses of systemic metabolism to determine the local versus systemic effects of social isolation on metabolism. We measured food consumption and

weight in isolated and grouped cohorts. Animal weights did not differ between isolated and group-housed mice prior to palpable tumor formation (age 10 wks, Table 1) or after tumor formation (age 17 wks, Table 1). However, isolated mice consumed more kilocalories per day compared to group-housed mice both before palpable tumor formation (age 8-10 wks; p=0.03, Table 1) and after (age 11-17 wks; p=0.0016, Table 1), suggesting a possible effect of social condition on eating behavior and/or systemic energy metabolism.

To test this possibility, parallel cohorts of chronically isolated and group-housed female SV40 Tag mice were placed in individual metabolic cages. This was also a test of the enduring effects of living in groups, as all mice had to be isolated during the metabolic cage studies because grouped metabolic cages are not available. When animals were placed in individual metabolic cages, we did not detect any systemic metabolic differences between the previously grouped and isolated cohorts (Sup. Fig.1 and Sup. Table 2). In addition, the previously observed differences in food consumption were no longer evident (p=0.80 active, p=0.27 inactive period; Table 1), suggesting that the superimposed stress of social isolation in metabolic cages affected the eating behavior of the grouped mice.

In addition to food consumption, animal weight, and metabolic cage analyses, we measured several circulating markers of systemic metabolism. As shown in Table 1, at 15 wks of age, we did not observe significant differences in circulating blood glucose, serum insulin, serum free-fatty acids, or serum leptin. Thus, circulating metabolic factor levels did not suggest a significant effect of social isolation on systemic metabolism.

**Upregulation of metabolic genes in the mammary gland occurs independently of the SV40Tag transgene and is not limited to the FVB/N mouse strain**

The SV40 Tag FVB/N mouse strain used in these experiments is a highly inbred transgenic cancer model. In addition, it is well-established that the FVB/N background is resistant to weight gain, suggesting an undefined metabolic phenotype (27). To rule out potential strain-specific or oncogene-associated effects of social isolation on either gene expression or systemic metabolism, we repeated the isolation versus group-housed studies using parental FVB/N (WT) and outbred CD-1 female mice.

We measured whole mammary gland gene expression from chronically isolated and group-housed 15-wk-old female WT FVB/N and CD-1 mice. The results recapitulated the upregulation of *Acaca* (WT, *p=0.03; CD-1, **p=0.01), *Hk2* (WT,

*p=0.02; CD-1, *p=0.09), and *Acly* (WT, ***p<0.001; CD-1, **p=0.007) steady-state mRNA that we observed in mammary glands from isolated 15-wk old SV40 Tag mice (Fig. 3A, C; Sup. Table 3). Moreover, as we had observed in the SV40 Tag mice, gene expression in gonadal fat was not significantly different between isolated versus group-housed female CD-1 mice (Fig. 3D, p>0.59 for all genes), and only one of the three metabolic genes was significantly upregulated in the WT FVB/N visceral fat (*Acaca* *p=0.04, *Hk2* p=0.47, *Acly* p=0.10; Fig. 3C). These results suggest that depot-specific upregulation of metabolic gene expression in mammary fat is a broader characteristic of mammary fat from chronically isolated mice.

**Upregulation of metabolic genes in mammary adipocytes results in elevated glucose and lipid metabolism.**

Phosphorylation of glucose by hexokinase 2 (encoded by *Hk2*) effectively traps glucose within cells for subsequent metabolism, including *de novo* lipid synthesis (lipogenesis). *Acly* and *Acaca* gene products also play essential roles in regulating lipid synthesis pathways (Fig. 4a). Therefore, we sought to determine whether the upregulation of *Hk2*, *Acly*, and *Acaca* in the mammary adipocytes of socially isolated animals resulted in the predicted functional increase in glucose metabolism and/or lipid synthesis.

Following 15 wks of either group housing or social isolation, we purified mammary adipocytes from individual SV40 Tag mouse mammary glands and measured their relative cellular glucose uptake from culture media in the presence (stimulated) or absence (basal) of insulin. Under basal and stimulated conditions, mammary adipocytes from socially isolated animals consumed roughly twice the amount of glucose compared to adipocytes from group-housed animals (Fig. 4B; *p=0.025, Wilcoxon rank sum test).

In a parallel experiment, we assessed the relative amount of glucose incorporated into lipid (a measurement of lipogenesis) in mammary adipocytes from the same set of animals. Under basal conditions, lipogenesis was nearly twice as high in mammary adipocytes from socially isolated mice (Fig. 4C; *p=0.05). Following insulin stimulation, the level of *de novo* lipid synthesis in isolated animals' mammary adipocytes further increased to roughly 3-fold higher than group-housed animals' adipocytes (Fig. 4C; *p=0.05, Wilcoxon rank sum test). Taken together, these results demonstrate that increased *Hk2*, *Acly*, and *Acaca* mRNA expression in mammary

adipocytes of socially isolated animals is associated with a concomitant increase in both glucose consumption and glucose incorporation into newly synthesized lipids.

**Leptin expression and secretion is increased in adipocytes from socially isolated animals.**

Both glucose metabolism and lipid synthesis have been proposed to be important regulators of intracellular content and secretion of the adipocyte chemokine, leptin (28, 29). Furthermore, previous *in vitro* and *in vivo* studies have implicated leptin in increased cancer cell proliferation and tumor growth (30-33) . Although there were no observed significant differences in circulating leptin between isolated and group-housed animals (Table 1), the metabolic changes we saw specific to mammary adipose tissue of socially isolated mice suggested that adipocytes may increase leptin secretion within the local mammary microenvironment. Therefore, we measured the leptin content of mammary adipocytes from socially isolated vs. group-housed mice.

Using both Western blot and ELISA, we observed roughly 60-70% more intracellular leptin in the mammary adipocytes of social isolates (Fig. 5A,B; Elisa, *p=0.01; Western blot, **p=0.009). To determine whether the elevation in intracellular leptin resulted in increased leptin secretion, we cultured mammary adipose tissue from isolated and group-housed animals for 24 hours under serum-free conditions. The media was then harvested and its leptin content was assessed. As observed in Fig. 5C, leptin levels were elevated in the mammary adipose tissue culture media from the socially isolated animals relative to media from group-housed mouse mammary adipose tissue (*p=0.02). Thus, social isolation appears to increase mammary adipocyte leptin protein expression and secretion.

**Mammary adipose tissue culture media potentiates the proliferation of SV40-Tag mammary epithelial cells *in vitro***

Adipose tissue depots are now considered to be endocrine organs, secreting numerous factors including leptin. Because we observed elevated leptin secretion from the mammary fat of socially isolated animals, we hypothesized that differential secretion of leptin and/or other adipokine factors contributes to the larger mammary tumor burden associated with social isolation. To evaluate the possibility that secreted factors from mammary fat contribute to cancer cell proliferation, we applied culture media from the leptin secretion experiments (Fig. 6) to an SV40-Tag-expressing mammary epithelial

cell line *in vitro*.(23). Media derived from culturing the mammary adipose tissue of socially isolated animals resulted in significantly more epithelial cell proliferation than media from group-housed mouse mammary adipose tissue (***$p<0.0001$). This observation supports the hypothesis that the local mammary adipocyte secretome contributes to cell proliferation and larger tumor growth seen in socially isolated versus group-housed mice.

**Key Research Accomplishments**

- We have Identified that metabolic gene changes associated with social isolation and increased tumor burden in female mice occur within the mammary adipocytes.
- We have determined that the metabolic gene expression changes in mammary adipocytes and associated with social isolation are not dependent on the background mouse strain.
- We have observed that gene expression changes are specific to the adipocytes of the mammary gland and are not observed in other fat depots.
- The gene expression changes observed in mammary adipocytes of socially isolated animals correlate with functional metabolic changes including increased glucose consumption and increased lipid synthesis.
- In addition to metabolic changes in mammary adipocytes of socially isolated animals, we have observed elevated levels and secretion of Leptin protein in isolated vs. grouped animals.
- Mammary fat conditioned media from social isolates potentiates the proliferation of cancer cells compared to media made using grouped animal mammary fat, suggesting adipocyte secreted proteins/metabolites are linked to the increased tumor burden observed *in vivo*.

## Reportable Outcomes

### Presentations:

**Mammary adipocyte-specific metabolic alterations are associated with paracrine effects on mammary tumorigenesis.** **Paul A. Volden**, Erin L. Wonder, Maxwell N. Skor, Christopher M. Carmean, Honggang Ye, Masha Kocherginsky, Eleanor Smith, Steven Kregel, Martha K. McClintock, Matthew J. Brady, and Suzanne D. Conzen. Keystone: Cancer metabolism, 2010, Banff, British Columbia

**Environmental stress and breast cancer biology: What is the link?** Conzen SD, **Volden PA**, Brady MJ, McClintock MK. AACR 2011: 102nd Annual Meeting, April 2-6 in Orlando, FL

### Publications:

Chronic social isolation is associated with metabolic gene expression changes specific to mammary adipose tissue. **Paul A. Volden**, Erin L. Wonder, Maxwell N. Skor, Christopher M. Carmean, Honggang Ye, Masha Kocherginsky, Steven Kregel, Martha K. McClintock, Matthew J. Brady, and Suzanne D. Conzen. In revision for Endocrinology

## Conclusion

Global gene expression data from our laboratory previously suggested a link between stress-induced mammary gland lipid synthesis gene expression and subsequently increased mammary tumor growth in the SV40 Tag model of ER-negative human breast cancer (7). Here we show that increased metabolic gene expression in mammary adipocytes from socially isolated mice is associated with a concomitant increase in mammary adipocyte glucose and lipid metabolism. Interestingly, the upregulation of *Acaca, Hk2,* and *Acly* steady-state mRNA that was involved in lipid synthesis, was not observed in the visceral fat depots of either FVB/N (both SV40-Tag and wild type) or CD-1 female mice. This suggests that stress-induced changes in metabolic gene expression are not strain-specific and are mammary adipocyte-specific. Furthermore, mammary adipocytes from socially isolated versus group-housed mice exhibited increased leptin production. When mammary fat was cultured, media from isolated animals' fat had significantly higher levels of secreted leptin and also stimulated SV40-Tag epithelial cell growth to a greater extent than media from group-housed adipose tissue. These results support the hypothesis that secreted factors from metabolically altered mammary adipose tissue contribute to larger tumor formation in socially isolated versus group-housed mice.

It is well-established that chronic exposure to unmitigated psychosocial stressors is correlated with an increased risk of metabolic and cardiovascular disease (34, 35). Similarly, chronic social isolation, an established psychosocial stressor for female rodents, is associated with increased mammary tumor growth in both the SV40 transgenic mouse (7) and Sprague-Dawley rat (8) models of breast cancer. More recent studies have also reported effects of psychosocial stress in rodent models of mammary tumorigenesis. Using a carcinogen-induced breast cancer model, Boyd et al. observed increased expression of ER-alpha and promotion of mammary tumorigenesis in adulthood when neonates were exposed to chronic, moderate psychosocial stress (36). In a more general mouse model of human cancer (p53 +/- mice), Hasen et al. reported a higher mortality rate in isolated female mice and, of the various types of cancers that arose in this model, breast cancer incidence was lower in the social isolates (37). These

investigations, which utilize highly divergent cancer models, all independently support a psychosocial influence on breast cancer biology.

The chronic stress of social isolation heightens and prolongs the acute glucocorticoid response to a superimposed, moderately severe stressor in rats (8). We recently demonstrated in female SV40-Tag mice that 30 minutes of physical restraint superimposed on chronic social isolation also increases the corticosterone stress response (7). We hypothesize that social isolation heightens the stress response to everyday stressors, including handling and the noises of routine animal husbandry, thereby exposing isolated mice to a significant increase in stress response pathways.

Interestingly, activation of these same neuroendocrine stress response pathways also regulates lipogenesis and fat utilization, as well as leptin expression (38-41). While we have yet to demonstrate that glucocorticoid and/or adrenergic signaling directly contribute to altered lipogenic gene expression in the mammary fat, it is tempting to speculate that these hormones likely influence metabolic gene expression. The finding that increased lipid metabolism-associated gene expression is significantly more prominent in mammary versus visceral fat was unanticipated. However, given the critical role of lactation in reproductive fitness, heightened transcriptional responsiveness of the mammary gland to stress hormones may allow adaptive milk production (42) and thereby favor offspring survival.

Adipocytes secrete numerous proteins, hormones and growth factors (adipokines), some of which have been linked to both inflammation (43, 44) and cancer progression (45-48). Thus, it is interesting that we observed elevated leptin secretion from the mammary fat of socially isolated animals. Indeed, several studies support a prominent role for leptin in breast cancer biology. For example, leptin has been observed to promote the proliferation of numerous breast cancer cell lines *in vitro (32)*. Furthermore, investigations using the MMTV PyMT mouse mammary tumor model with an intact central leptin signaling pathway, but deficient in peripheral leptin receptor expression, resulted in attenuated tumor progression, providing strong evidence for local mammary leptin action as a critical mediator of tumor growth (49). Leptin has also been reported to upregulate VEGF secretion in human and mouse mammary tumor cells (30); thus, leptin action may influence tumor growth through roles as both a growth and an angiogenic factor.

Similar to our model, environment-associated changes in leptin regulation have been observed in both humans and rodents. For example, serum leptin levels were elevated in men classified as socially isolated and depressed (50). In male mice, Cao et al. suggested that an "enriched" housing environment results in decreased tumor growth through lowered systemic levels of leptin (51). Thus, it is intriguing that in two recently reported and highly diverse mouse models of cancer (7, 50) an association between the social environment, changes in fat metabolism, and altered cancer growth has been identified.

We hypothesize that increased local leptin secretion from socially isolated animals' mammary adipocytes contributes to promoting mammary tumor growth. However, the elevated adipocyte leptin production and secretion may also parallel changes in additional adipocyte-secreted factors. Adipokines, such as adiponectin, resistin, and collagen VI have also been reported to influence tumor biology (52-57) and a potential role for these and other factors in our model cannot be excluded. Furthermore, social isolation of female rodents is associated with a heightened glucocorticoid response, and it is known that glucocorticoids can act directly on the mammary epithelium. *In vivo*, glucocorticoids may promote tumor growth, though previous data suggest a predominant role of glucocorticoids in inhibiting apoptosis rather than increasing proliferation (58). In future studies, we will perform experiments to

clarify the role of glucocorticoids in socially isolated mammary tumor growth, as well as to determine the specific factor(s) secreted by mammary adipocytes that may influence tumor growth. Based on the findings of this study, we propose a model wherein social isolation and its ensuing neuroendocrine effects modify mammary adipocyte metabolism and associated adipokine secretion, thereby potentiating estrogen-independent mammary tumor growth (Fig.7).

## References


1. **Berkman LF, Syme SL** 1979 Social networks, host-resistance, and mortality - 9-year follow-up-study of alameda county residents. American Journal of Epidemiology 109:186-204
2. **Duijts SFA, Zeegers MPA, Van der Borne B** 2003 The association between stressful life events and breast cancer risk: A meta-analysis. International Journal of Cancer 107:1023-1029
3. **Stefanek ME, Palmer SC, Thombs BD, Coyne JC** 2009 Finding What Is Not There Unwarranted Claims of an Effect of Psychosocial Intervention on Recurrence and Survival. Cancer 115:5612-5616
4. **Andersen BL, Yang HC, Farrar WB, Golden-Kreutz DM, Emery CF, Thornton LM, Young DC, Carson WE** 2008 Psychologic Intervention Improves Survival for Breast Cancer Patients A Randomized Clinical Trial. Cancer 113:3450-3458
5. **Williams JB, Pang D, Delgado B, Kocherginsky M, Tretiakova M, Krausz T, Pan D, He J, McClintock MK, Conzen SD** 2009 A Model of Gene-Environment Interaction Reveals Altered Mammary Gland Gene Expression and Increased Tumor Growth following Social Isolation. Cancer Prevention Research 2:850-861
6. **Hermes GL, Delgado B, Tretiakova M, Cavigelli SA, Krausz T, Conzen SD, McClintock MK** 2009 Social isolation dysregulates endocrine and behavioral stress while increasing malignant burden of spontaneous mammary tumors. Proceedings of the National Academy of Sciences of the United States of America 106:22393-22398
7. **Kaur B, Jorgensen A, Duttaroy AK** 2009 Fatty acid uptake by breast cancer cells (MDA-MB-231): Effects of insulin, leptin, adiponectin, and TNF alpha. Prostaglandins Leukotrienes and Essential Fatty Acids 80:93-99
8. **Munoz-de-Toro M, Durando M, Beldomenico PM, Beldomenico HR, Kass L, Garcia SR, Luque EH** 2006 Estrogenic microenvironment generated by organochlorine residues in adipose mammary tissue modulates biomarker expression in ER alpha-positive breast carcinomas. Breast Cancer Research 8
9. **Brunet J, Vazquez-Martin A, Colomer R, Grana-Suarez B, Martin-Castillo B, Menendez JA** 2008 BRCA1 and acetyl-CoA carboxylase: The metabolic syndrome of breast cancer. Molecular Carcinogenesis 47:157-163
10. **Taylor A, Cheng KK** 2003 Social deprivation and breast cancer. Journal of Public Health Medicine 25:228-233
11. **Daling JR, Malone KE, Doody DR, Johnson LG, Gralow JR, Porter PL** 2001 Relation of body mass index to tumor markers and survival among young women with invasive ductal breast carcinoma. Cancer 92:720-729
12. **Bartolomucci A, Cabassi A, Govoni P, Ceresini G, Cero C, Berra D, Dadomo H, Franceschini P, Dell'Omo G, Parmigiani S, Palanza P** 2009 Metabolic Consequences and Vulnerability to Diet-Induced Obesity in Male Mice under Chronic Social Stress. Plos One 4
13. **Chuang JC, Cui HX, Mason BL, Mahgoub M, Bookout AL, Yu HG, Perello M, Elmquist JK, Repa JJ, Zigman JM, Lutter M** 2010 Chronic social defeat stress disrupts regulation of lipid synthesis. Journal of Lipid Research 51:1344-1353
14. **Guallar-Castillon P, Balboa-Castillo T, Lopez-Garcia E, Leon-Munoz LM, Gutierrez-Fisac JL, Banegas JR, Rodriguez-Artalejo F** 2009 BMI, Waist Circumference, and Mortality According to Health Status in the Older Adult Population of Spain. Obesity 17:2232-2238

15. **Nonogaki K, Nozue K, Oka Y** 2007 Social isolation affects the development of obesity and type 2 diabetes in mice. Endocrinology 148:4658-4666
16. **Kim JB, Stein R, O'Hare MJ** 2005 Tumour-stromal interactions in breast cancer: The role of stroma in tumourigenesis. Tumor Biology 26:173-185
17. **Scherer PE** 2006 Adipose tissue - From lipid storage compartment to endocrine organ. Diabetes 55:1537-1545
18. **Park J, Euhus DM, Scherer PE** 2011 Paracrine and Endocrine Effects of Adipose Tissue on Cancer Development and Progression. Endocr Rev
19. **Cirillo D, Rachiglio AM, la Montagna R, Giordano A, Normanno N** 2008 Leptin Signaling in Breast Cancer: An Overview. Journal of Cellular Biochemistry 105:956-964
20. **Wajchenberg BL** 2000 Subcutaneous and visceral adipose tissue: Their relation to the metabolic syndrome. Endocrine Reviews 21:697-738
21. **Cavigelli SA, Yee JR, McClintock MK** 2006 Infant temperament predicts life span in female rats that develop spontaneous tumors. Hormones and Behavior 50:454-462
22. **Yuen VG, McNeill JH** 2000 Comparison of the glucose oxidase method for glucose determination by manual assay and automated analyzer. Journal of Pharmacological and Toxicological Methods 44:543-546
23. **Holzer RG, MacDougall C, Cortright G, Atwood K, Green JE, Jorcyk CL** 2003 Development and characterization of a progressive series of mammary adenocarcinoma cell lines derived from the C3(1)/SV40 Large T-antigen transgenic mouse model. Breast Cancer Research and Treatment 77:65-76
24. **Vichai V, Kirtikara K** 2006 Sulforhodamine B colorimetric assay for cytotoxicity screening. Nature Protocols 1:1112-1116
25. **Green JE, Shibata MA, Yoshidome K, Liu ML, Jorcyk C, Anver MR, Wigginton J, Wiltrout R, Shibata E, Kaczmarczyk S, Wang W, Liu ZY, Calvo A, Couldrey C** 2000 The C3(1)/SV40 T-antigen transgenic mouse model of mammary cancer: ductal epithelial cell targeting with multistage progression to carcinoma. Oncogene 19:1020-1027
26. **Jurczak MJ, Danos AM, Rehrmann VR, Allison MB, Greenberg CC, Brady MJ** 2007 Transgenic overexpression of protein targeting to glycogen markedly increases adipocytic glycogen storage in mice. American Journal of Physiology-Endocrinology and Metabolism 292:E952-E963
27. **La Merrill M, Baston DS, Denison MS, Birnbaum LS, Pomp D, Threadgill DW** 2009 Mouse breast cancer model-dependent changes in metabolic syndrome-associated phenotypes caused by maternal dioxin exposure and dietary fat. American Journal of Physiology-Endocrinology and Metabolism 296:E203-E210
28. **Walker CG, Bryson JM, Hancock DP, Caterson ID** 2007 Leptin secretion is related to glucose-derived lipogenesis in isolated adipocytes. International Journal of Obesity 31:723-729
29. **Mueller WM, Gregoire FM, Stanhope KL, Mobbs CV, Mizuno TM, Warden CH, Stern JS, Havel PJ** 1998 Evidence that glucose metabolism regulates leptin secretion from cultured rat adipocytes. Endocrinology 139:551-558
30. **Gonzalez-Perez RR, Xu YB, Guo SC, Watters A, Zhou WQ, Leibovich SJ** 2010 Leptin upregulates VEGF in breast cancer via canonic and non-canonical signalling pathways and NF kappa B/HIF-1 alpha activation. Cellular Signalling 22:1350-1362
31. **Gonzalez RR, Watters A, Xu YB, Singh UP, Mann DR, Rueda BR, Penichet ML** 2009 Leptin-signaling inhibition results in efficient anti-tumor activity in estrogen receptor positive or negative breast cancer. Breast Cancer Research 11

32. **Ray A, Nkhata KJ, Cleary MP** 2007 Effects of leptin on human breast cancer cell lines in relationship to estrogen receptor and HER2 status. International Journal of Oncology 30:1499-1509
33. **Hu X, Juneja SC, Maihle NJ, Cleary MGP** 2002 Leptin - A growth factor in normal and malignant breast cells and for normal mammary gland development. Journal of the National Cancer Institute 94:1704-1711
34. **Brunner EJ, Hemingway H, Walker BR, Page M, Clarke P, Juneja M, Shipley MJ, Kumari M, Andrew R, Seckl JR, Papadopoulos A, Checkley S, Rumley A, Lowe GDO, Stansfeld SA, Marmot MG** 2002 Adrenocortical, autonomic, and inflammatory causes of the metabolic syndrome - Nested case-control study. Circulation 106:2659-2665
35. **Ginzburg K, Wrensch M, Rice T, Farren G, Spiegel D** 2008 Breast cancer and psychosocial factors: Early stressful life events, social support, and well-being. Psychosomatics 49:407-412
36. **Boyd AL, Salleh A, Humber B, Yee J, Tomes L, Kerr LR** 2010 Neonatal Experiences Differentially Influence Mammary Gland Morphology, Estrogen Receptor alpha Protein Levels, and Carcinogenesis in BALB/c Mice. Cancer Prevention Research 3:1398-1408
37. **Hasen NS, O'Leary KA, Auger AP, Schuler LA** 2010 Social Isolation Reduces Mammary Development, Tumor Incidence, and Expression of Epigenetic Regulators in Wild-type and p53-Heterozygotic Mice. Cancer Prevention Research 3:620-629
38. **Diamant S, Shafrir E** 1975 MODULATION OF ACTIVITY OF INSULIN-DEPENDENT ENZYMES OF LIPOGENESIS BY GLUCOCORTICOIDS. European Journal of Biochemistry 53:541-546
39. **Bhathena SJ** 2006 Relationship between fatty acids and the endocrine and neuroendocrine system. Nutritional Neuroscience 9:1-10
40. **Lewandowski K, Randeva HS, O'Callaghan CJ, Horn R, Medley GF, Hillhouse EW, Brabant G, O'Hare P** 2001 Effects of insulin and glucocorticoids on the leptin system are mediated through free leptin. Clinical Endocrinology 54:533-539
41. **Slieker LJ, Sloop KW, Surface PL, Kriauciunas A, LaQuier F, Manetta J, BueValleskey J, Stephens TW** 1996 Regulation of expression of ob mRNA and protein by glucocorticoids and cAMP. Journal of Biological Chemistry 271:5301-5304
42. **Hovey RC, Aimo L** 2010 Diverse and Active Roles for Adipocytes During Mammary Gland Growth and Function. Journal of Mammary Gland Biology and Neoplasia 15:279-290
43. **Faggioni R, Feingold KR, Grunfeld C** 2001 Leptin regulation of the immune response and the immunodeficiency of malnutrition. Faseb Journal 15:2565-2571
44. **Otero M, Lago R, Gomez R, Lago F, Dieguez C, Gomez-Reino JJ, Gualillo O** 2006 Changes in plasma levels of fat-derived hormones adiponectin, leptin, resistin and visfatin in patients with rheumatoid arthritis. Annals of the Rheumatic Diseases 65:1198-1201
45. **Byeon JS, Jeong JY, Kim MJ, Lee SM, Nam WH, Myung SJ, Kim JG, Yang SK, Kim JH, Suh DJ** 2010 Adiponectin and adiponectin receptor in relation to colorectal cancer progression. International Journal of Cancer 127:2758-2767
46. **Riolfi M, Ferla R, Del Valle L, Pina-Oviedo S, Scolaro L, Micciolo R, Guidi M, Terrasi M, Cetto GL, Surmacz E** 2010 Leptin and Its Receptor are Overexpressed in Brain Tumors and Correlate with the Degree of Malignancy. Brain Pathology 20:481-489

47. **Jarde T, Caldefie-Chezet F, Damez M, Mishellany F, Penault-Llorca F, Guillot J, Vasson MP** 2008 Leptin and leptin receptor involvement in cancer development: A study on human primary breast carcinoma. Oncology Reports 19:905-911
48. **Iyengar P, Combs TP, Shah SJ, Gouon-Evans V, Pollard JW, Albanese C, Flanagan L, Tenniswood MP, Guha C, Lisanti MP, Pestell RG, Scherer PE** 2003 Adipocyte-secreted factors synergistically promote mammary tumorigenesis through induction of anti-apoptotic transcriptional programs and proto-oncogene stabilization. Oncogene 22:6408-6423
49. **Park J, Kusminski CM, Chua SC, Scherer PE** 2010 Leptin Receptor Signaling Supports Cancer Cell Metabolism through Suppression of Mitochondrial Respiration in Vivo. American Journal of Pathology 177:3133-3144
50. **Häfner S, Zierer A, Emeny RT, Thorand B, Herder C, Koenig W, Rupprecht R, Ladwig KH, Investigators KS** 2011 Social isolation and depressed mood are associated with elevated serum leptin levels in men but not in women. Psychoneuroendocrinology 36:200-209
51. **Cao L, Liu XL, Lin EJD, Wang CS, Choi EY, Riban V, Lin B, During MJ** 2010 Environmental and Genetic Activation of a Brain-Adipocyte BDNF/Leptin Axis Causes Cancer Remission and Inhibition. Cell 142:52-64
52. **Wang Y, Lam JB, Lam KSL, Liu J, Lam MC, Hoo RLC, Wu DH, Cooper GJS, Xu AM** 2006 Adiponectin modulates the glycogen synthase kinase-3 beta/beta-catenin signaling pathway and attenuates mammary tumorigenesis of MDA-MB-231 cells in nude mice. Cancer Research 66:11462-11470
53. **Li G, Cong L, Gasser J, Zhao J, Chen K, Li FH** 2011 Mechanisms Underlying the Anti-Proliferative Actions of Adiponectin in Human Breast Cancer Cells, MCF7-Dependency on the cAMP/Protein Kinase-A Pathway. Nutrition and Cancer-an International Journal 63:80-88
54. **Pfeiler GH, Buechler C, Neumeier M, Schaffler A, Schmitz G, Ortmann O, Treeck O** 2008 Adiponectin effects on human breast cancer cells are dependent on 17-ss estradiol. Oncology Reports 19:787-793
55. **Dalamaga M, Karmaniolas K, Panagiotou A, Hsi A, Chamberland J, Dimas C, Lekka A, Mantzoros CS** 2009 Low circulating adiponectin and resistin, but not leptin, levels are associated with multiple myeloma risk: a case-control study. Cancer Causes & Control 20:193-199
56. **Sun CA, Wu MH, Chu CH, Chou YC, Hsu GC, Yang T, Chou WY, Yu CP, Yu JC** 2010 Adipocytokine resistin and breast cancer risk. Breast Cancer Research and Treatment 123:869-876
57. **Iyengar P, Espina V, Williams TW, Lin Y, Berry D, Jelicks LA, Lee H, Temple K, Graves R, Pollard J, Chopra N, Russell RG, Sasisekharan R, Trock BJ, Lippman M, Calvert VS, Petricoin EF, Liotta L, Dadachova E, Pestell RG, Lisanti MP, Bonaldo P, Scherer PE** 2005 Adipocyte-derived collagen VI affects early mammary tumor progression in vivo, demonstrating a critical interaction in the tumor/stroma microenvironment. Journal of Clinical Investigation 115:1163-1176
58. **Wu W, Chaudhuri S, Brickley DR, Pang D, Karrison T, Conzen SD** 2004 Microarray analysis reveals glucocorticoid-regulated survival genes that are associated with inhibition of apoptosis in breast epithelial cells. Cancer Research 64:1757-1764

## Appendices

### Figure Legends

**Fig. 1.** Tumor and behavior measurements from isolated and group housed mice between 11-17 weeks of age. A, Relative palpable tumor incidence of isolated and grouped mice 11-17 weeks. B, Average tumor volume per affected mouse by 18 weeks of age: socially isolated vs. group-housed mice (*p<0.01; Wilcoxon rank sum test). C, Isolated mice displayed a greater vigilance (longer latency to leave home base), compared to group-housed mice (p<0.0001; log-rank test; 16 weeks of age).

**Fig. 2.** Mammary gland cell fractionation followed by Q-RT-PCR measurement of gene expression from isolated and group-housed mice. *A,* Collagenase treatment followed by centrifugation was used to separate SV40 Tag mammary gland adipocytes (left panel) from the non-adipocytes (epithelial/stromal cells, right panel). B, Relative *Hk2, Acly,* and *Acaca* gene expression in the adipocytes (white bars) vs. non-adipocyte (gray) cells. Expression of *Hk2, Acly,* and *Acaca* normalized to beta-actin (*Actb*) mRNA expression in mammary adipocytes (C), non-adipocytes (epithelial/stromal cells, D), and in visceral fat (E) in isolated (white bars) vs. grouped mice (gray). Error bars indicate 95% confidence intervals. In panels C-E; *p<0.05, ***p<0.001, NS=not significant.

**Fig. 3.** Relative mammary gland and visceral fat depot gene expression in differentially housed WT-FVB/N and CD1 mice**.** *Hk2, Acly,* and *Acaca* gene expression from 15 week old WT-FVB/N mammary glands (A) or WT FVB/N gonadal adipose tissue (B). *Hk2, Acly,* and *Acaca* gene expression at 15 weeks in CD-1 mice mammary glands (C) or in CD-1 mice gonadal adipose tissue (D). Socially Isolated mRNA expression (white bars) relative to group-housed mRNA expression (gray). Error bars indicate 95% confidence intervals. In all panels; *p<0.05, ***p<0.001, NS=not significant.

**Fig. 4.** Glucose consumption and lipogenesis in isolated mammary adipocytes from differentially housed SV40-Tag mice**.** A, *Hk2, Acly,* and *Acaca* gene products involved in glucose and lipid metabolism. B, mammary adipocytes from group housed and socially isolated animals were cultured in low glucose media, +/- insulin, for 4 hours. Media was then harvested and glucose in the media was measured via the glucose oxidase method and consumption assessed by a comparison to glucose in fresh (not used for culture) media. C, mammary adipocytes were purified from group housed and socially isolated animals and immediately cultured with $^{14}C$ glucose, +/- insulin, for 1hr.

The lipid fraction was extracted from the adipocytes and $^{14}C$, representing the incorporation of labeled glucose into lipid species, was counted using a scintillation counter. *p≤0.05, Wilcoxon rank sum test.

**Fig. 5.** Leptin content in mammary adipocyte lysates and mammary tissue culture media from differentially housed mice. A, Representative Western blot analysis of mammary adipocyte leptin, adiponectin, and actin expression from grouped and socially isolated animals. B, Relative mammary adipocyte leptin from tissue lysates as measured by Western blot densitometry and ELISA. C, Mammary adipocyte secreted leptin in 24hr culture media, as measured by ELISA. Error bars indicate standard deviation. *p<0.05, **p<0.01, Wilcoxon rank sum test.

**Fig. 6.** Proliferative effects of serum free media following 24hr mammary adipose tissue culture from differentially housed SV40Tag mice. Media was harvested and applied to an SV40-Tag mammary epithelial cell line and cell number over time was measured using the SRB assay. Serum free DMEM with 1%BSA was used as a control. ***p<0.0001 isolated vs. grouped.

**Fig. 7.** Diagram of current model connecting the social environment, stress response, and the neuroendocrine axis with increased mammary tumor burden in part through changes in mammary adipocyte biology.

**Table 1.** Measurements of circulating metabolic parameters, food consumption, and weights in grouped vs. isolated SV40-Tag female mice. Circulating markers were obtained from 15wk old animals. Food consumption and weights are divided into pre- and post-tumor time periods to investigate the effects of tumor burden on metabolism. Data indicate means +/- standards deviation, with p-value obtained using a student's T-test.

Fig. 1

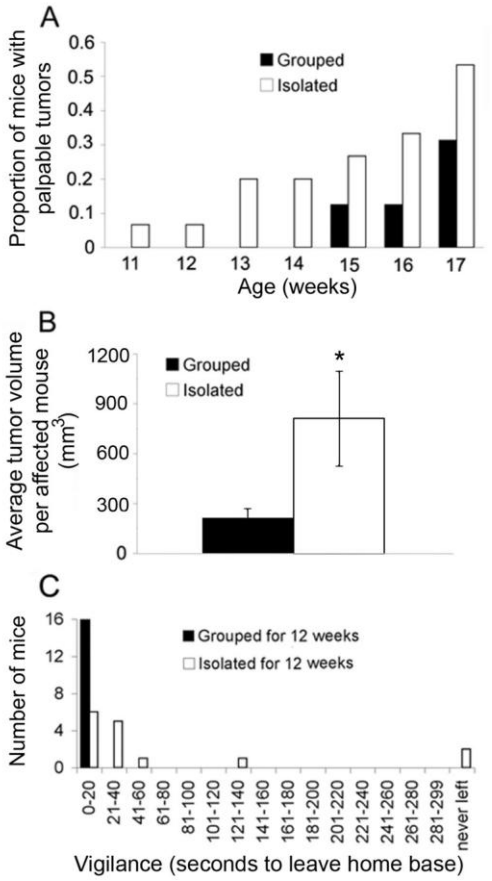

A
Proportion of mice with palpable tumors
0.6
0.5
0.4
0.3
0.2
0.1
0
Grouped
Isolated
11
12
13
14
15
16
17
Age (weeks)
B
Average tumor volume per affected mouse (mm3)
1200
900
600
300
0
Grouped
Isolated
*
C
Number of mice
16
12
8
4
0
Grouped for 12 weeks
Isolated for 12 weeks
0-20
21-40
41-60
61-80
81-100
101-120
121-140
141-160
161-180
181-200
201-220
221-240
241-260
261-280
281-299
never left
Vigilance (seconds to leave home base)

**Fig. 2**

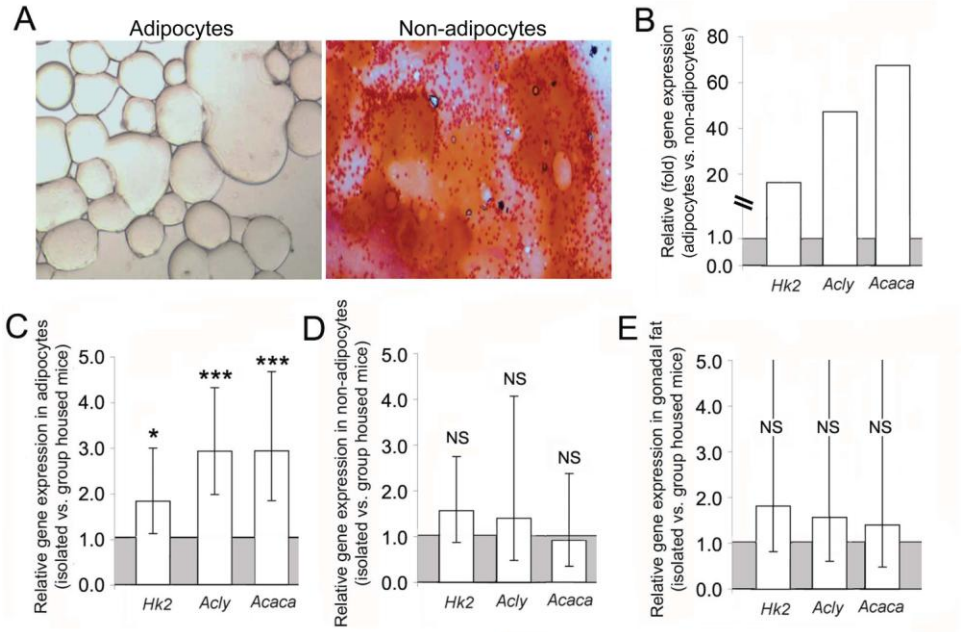

A
Adipocytes
Non-adipocytes
B
Relative (fold) gene expression (adipocytes vs. non-adipocytes)
80
60
40
20
1.0
0.0
Hk2
Acly
Acaca
C
Relative gene expression in adipocytes (isolated vs. group housed mice)
5.0
4.0
3.0
2.0
1.0
0.0
*
***
***
Hk2
Acly
Acaca
D
Relative gene expression in non-adipocytes (isolated vs. group housed mice)
NS
NS
NS
E
Relative gene expression in gonadal fat (isolated vs. group housed mice)
NS
NS
NS

**Fig. 3**

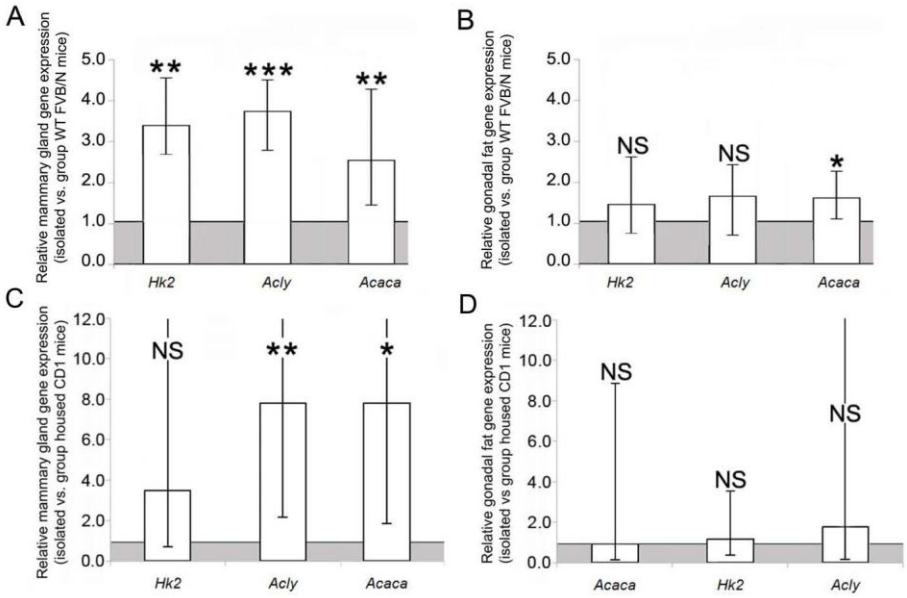

A
Relative mammary gland gene expression (isolated vs. group WT FVB/N mice)
5.0
4.0
3.0
2.0
1.0
0.0
**
***
**
Hk2
Acly
Acaca
B
Relative gonadal fat gene expression (isolated vs. group WT FVB/N mice)
NS
NS
*
Hk2
Acly
Acaca
C
Relative mammary gland gene expression (isolated vs. group housed CD1 mice)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
NS
**
*
Hk2
Acly
Acaca
D
Relative gonadal fat gene expression (isolated vs group housed CD1 mice)
NS
NS
NS
Acaca
Hk2
Acly

**Fig. 4**

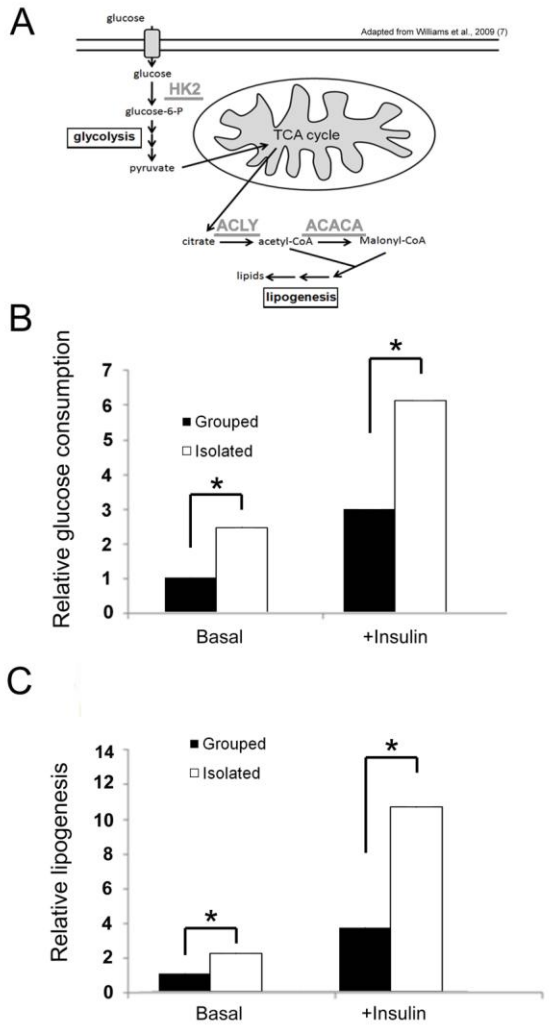

A
glucose
Adapted from Williams et al., 2009 (7)
glucose
HK2
glucose-6-P
glycolysis
pyruvate
TCA cycle
ACLY
ACACA
citrate
acetyl-CoA
Malonyl-CoA
lipids
lipogenesis
B
Relative glucose consumption
7
6
5
4
3
2
1
0
Grouped
Isolated
*
*
Basal
+Insulin
C
Relative lipogenesis
14
12
10
8
6
4
2
0
Grouped
Isolated
*
*
Basal
+Insulin

**Fig. 5**

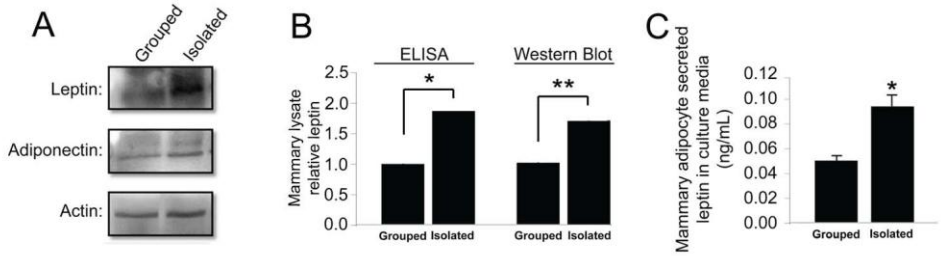

A
Grouped
Isolated
Leptin:
Adiponectin:
Actin:
B
ELISA
Western Blot
Mammary lysate relative leptin
2.5
2.0
1.5
1.0
0.5
0.0
*
**
Grouped
Isolated
Grouped
Isolated
C
Mammary adipocyte secreted leptin in culture media (ng/mL)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
*
Grouped
Isolated

**Fig. 6**

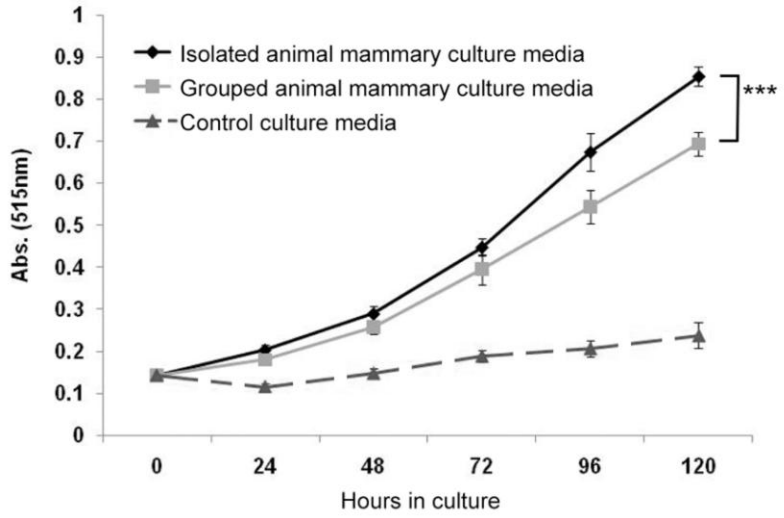

Isolated animal mammary culture media
Grouped animal mammary culture media
Control culture media
***
Abs. (515nm)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
24
48
72
96
120
Hours in culture

**Fig. 7**

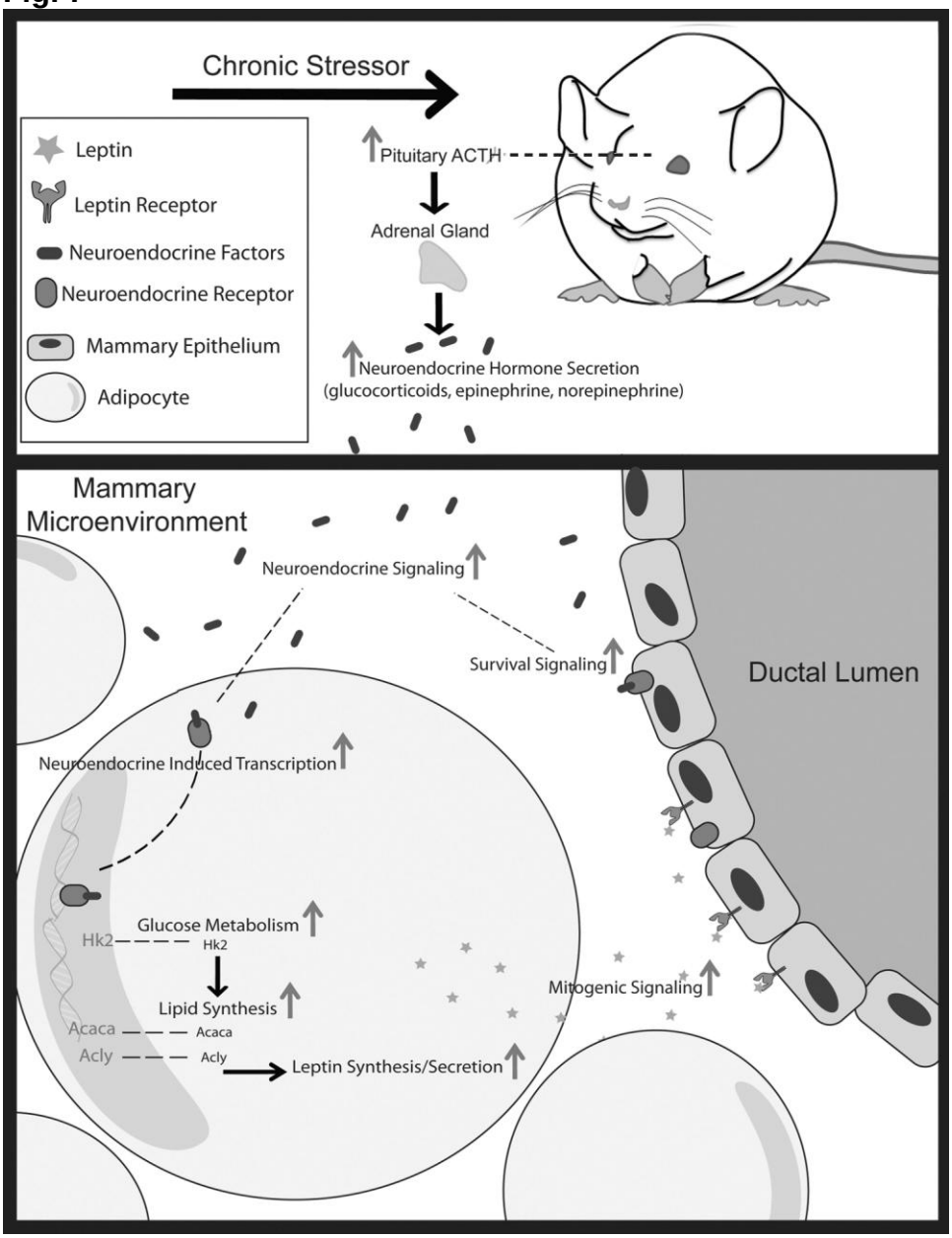

Chronic Stressor
Leptin
Leptin Receptor
Neuroendocrine Factors
Neuroendocrine Receptor
Mammary Epithelium
Adipocyte
Pituitary ACTH
Adrenal Gland
Neuroendocrine Hormone Secretion
(glucocorticoids, epinephrine, norepinephrine)
Mammary Microenvironment
Neuroendocrine Signaling
Survival Signaling
Ductal Lumen
Neuroendocrine Induced Transcription
Glucose Metabolism
Hk2
Hk2
Lipid Synthesis
Acaca
Acaca
Acly
Acly
Leptin Synthesis/Secretion
Mitogenic Signaling

**Table 1**

| | Grouped | Isolated | p-value |
|---|---|---|---|
| Blood Glucose (mg/dL) | 113.4 ± 9.85 | 110.4 ± 3.54 | 0.79 |
| Serum Insulin (pg/mL) | 361.5 ± 78.8 | 349.3 ± 82.8 | 0.92 |
| NEFA (mEq/L) | 0.93 ± 0.10 | 0.79 ± 0.07 | 0.24 |
| Serum Leptin (ng/mL) | 1.36 ± 0.39 | 1.31 ± 0.55 | 0.94 |
| Food consumption: 8-10wks (kcal/day) | 9.33 ± 0.36 | 10.60 ± 0.26 | 0.03 |
| Food consumption: 11-17wks (kcal/day) | 10.47 ± 0.35 | 12.58 ± 0.35 | 0.002 |
| Weight: age 10wks (g) | 18.9 ± 0.48 | 18.8 ± 0.23 | 0.85 |
| Weight: age 17wks (g) | 21.2 ± 0.47 | 21.4 ± 0.54 | 0.81 |

**Supp. Table 1: SV40 Tag Q-RT-PCR analysis**

| Label | Gene | Pvalue | $2^{-\Delta\Delta Ct}$ | 95% CI |
|---|---|---|---|---|
| SV40 Mamm Adipo | *acaca* | <.0001 | 2.94 | 1.85-4.68 |
| SV40 Mamm Adipo | *hk2* | 0.0167 | 1.84 | 1.13-3.00 |
| SV40 Mamm Adipo | *acly* | <.0001 | 2.93 | 1.99-4.33 |
| SV40 Mamm Epithel | *acaca* | 0.8559 | 0.92 | 0.35-2.39 |
| SV40 Mamm Epithel | *hk2* | 0.1167 | 1.56 | 0.89-2.75 |
| SV40 Mamm Epithel | *acly* | 0.5284 | 1.4 | 0.48-4.07 |
| SV40 Gonad | *acaca* | 0.8243 | 1.3 | 0.09-18.36 |
| SV40 Gonad | *hk2* | 0.4298 | 1.89 | 0.32-11.09 |
| SV40 Gonad | *acly* | 0.6345 | 1.72 | 0.14-21.4 |

**Supp. Table 2: FVB/N-WT and CD1 gene expression analysis**

| Label | Gene | Pvalue | $2^{-\Delta\Delta Ct}$ | 95% CI |
|---|---|---|---|---|
| FVB/N WT Mamm | *acaca* | 0.0292 | 2.42 | 1.11-5.28 |
| FVB/N WT Mamm | *hk2* | 0.0215 | 3.36 | 1.22-9.25 |
| FVB/N WT Mamm | *acly* | 0.0006 | 3.71 | 1.91-7.12 |
| FVB/N WT Gonad | *acaca* | 0.0373 | 1.7 | 1.04-2.79 |
| FVB/N WT Gonad | *hk2* | 0.0994 | 1.2 | 0.72-2.01 |
| FVB/N WT Gonad | *acly* | 0.4699 | 1.81 | 0.88-3.71 |
| | | | | |
| CD-1 Mamm | *acaca* | 0.0117 | 7.78 | 1.85-32.68 |
| CD-1 Mamm | *hk2* | 0.0941 | 3.48 | 0.76-15.99 |
| CD-1 Mamm | *acly* | 0.007 | 7.79 | 2.15-28.27 |
| CD-1 Gonad | *acaca* | 0.9923 | 0.99 | 0.11-8.82 |
| CD-1 Gonad | *hk2* | 0.7277 | 1.19 | 0.36-3.89 |
| CD-1 Gonad | *acly* | 0.5887 | 1.82 | 0.13-26.45 |

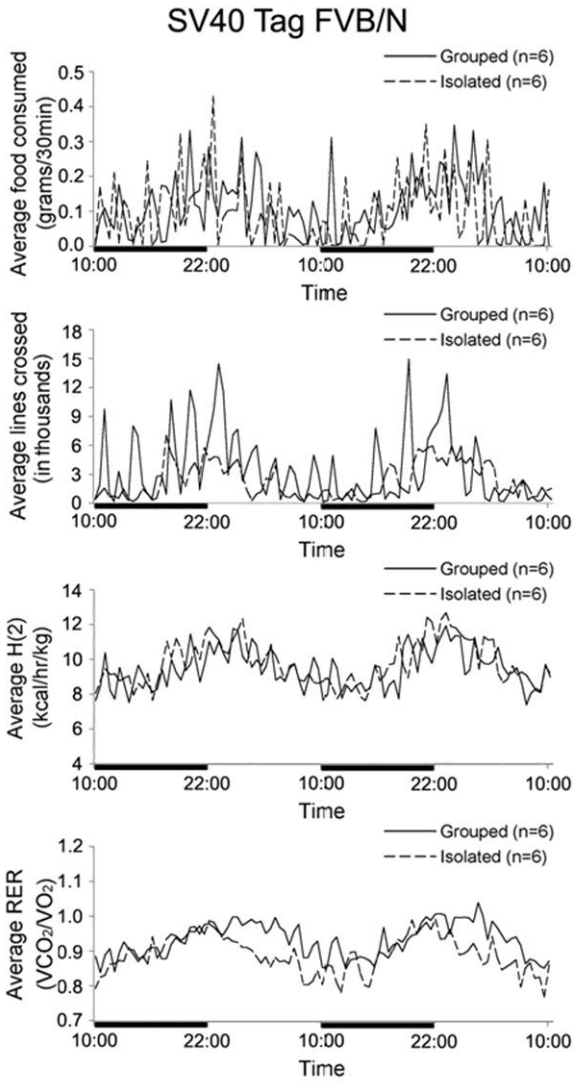
**Supp. Table 3: Metabolic cage measurements of isolated vs. grouped SV40-Tag Mice**

| | Active Period | | | Inactive Period | | |
|---|---|---|---|---|---|---|
| **Metabolic Parameter*** | **Isolated (n=6)** | **Grouped (n=6)** | **p value** | **Isolated (n=6)** | **Grouped (n=6)** | **p value** |
| Food consumed (g) | 0.12 ± 0.01 | 0.13 ± 0.02 | 0.80 | 0.05 ± 0.00 | 0.06 ± 0.01 | 0.27 |
| Motor activity (lines crossed) | 3797 ± 1228 | 3174 ± 1955 | 0.80 | 925 ± 107 | 1392 ± 714 | 0.55 |
| Energy expenditure (kcal/hr/kg) | 10.44 ± 0.55 | 9.90 ± 0.51 | 0.50 | 8.53 ± 0.30 | 8.59 ± 0.28 | 0.89 |
| Carb/fat ratio RER ($VCO_2/VO_2$) | 0.90 ± 0.01 | 0.94 ± 0.02 | 0.21 | 0.85 ± 0.01 | 0.86 ± 0.02 | 0.42 |

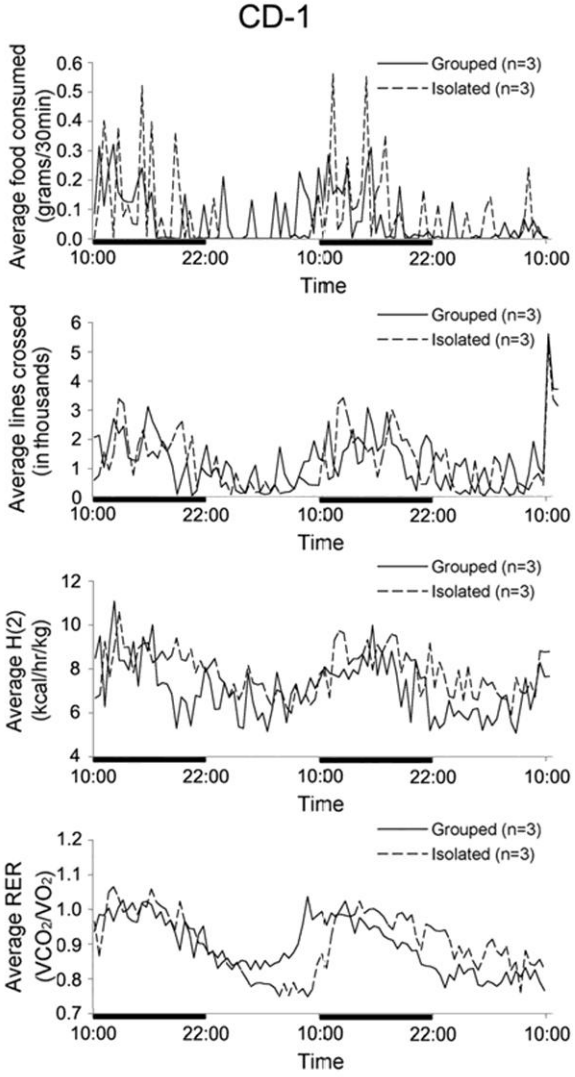
**Supp. Table 4: Metabolic cage measurements of isolated vs. grouped CD-1 Mice**

| | Active Period | | | Inactive Period | | |
|---|---|---|---|---|---|---|
| **Metabolic Parameter*** | **Isolated (n=3)** | **Grouped (n=3)** | **P value** | **Isolated (n=3)** | **Grouped (n=3)** | **P value** |
| Food consumed (g) | 0.12 ± 0.02 | 0.10 ± 0.01 | 0.40 | 0.02 ± 0.00 | 0.04 ± 0.01 | 0.11 |
| Motor activity (lines crossed) | 1660 ± 323 | 1564 ± 213 | 0.82 | 706 ± 121 | 989 ± 71 | 0.13 |
| Energy expenditure (kcal/hr/kg) | 8.46 ± 0.18 | 7.93 ± 0.26 | 0.18 | 7.23 ± 0.24 | 6.28 ± 0.15 | 0.37 |
| Carb/fat ratio RER ($VCO_2/VO_2$) | 0.97 ± 0.02 | 0.95 ± 0.01 | 0.27 | 0.84 ± 0.01 | 0.84 ± 0.01 | 0.98 |

**Supp. Fig.1**


SV40 Tag FVB/N
Grouped (n=6)
Isolated (n=6)
Average food consumed (grams/30min)
0.5
0.4
0.3
0.2
0.1
0.0
10:00
22:00
10:00
22:00
10:00
Time
Grouped (n=6)
Isolated (n=6)
Average lines crossed (in thousands)
18
15
12
9
6
3
0
10:00
22:00
10:00
22:00
10:00
Time
Grouped (n=6)
Isolated (n=6)
Average H(2) (kcal/hr/kg)
14
12
10
8
6
4
10:00
22:00
10:00
22:00
10:00
Time
Grouped (n=6)
Isolated (n=6)
Average RER ($VCO_2/VO_2$)
1.2
1.1
1.0
0.9
0.8
0.7
10:00
22:00
10:00
22:00
10:00
Time

**Supp. Fig.2**




**Supplemental Table 1.** SV40-Tag gene expression analysis. A summary of the raw Q-RT-PCR data obtained from isolated and grouped SV40-Tag animals' gonadal fat and from cells following mammary gland fractionation. Data are depicted in figure 2.

**Supplemental Table 2:** FVB/N-WT and CD-1 gene expression analysis. A summary of the raw Q-RT-PCR data obtained from isolated and grouped FVB/N-WT and CD-1 animals' mammary glands and gonadal fat and depicted in figure 3.

**Supplemental Table 3:** Metabolic cage measurements of isolated vs. grouped SV40-Tag mice. A summary of the metabolic cage analysis for grouped and isolated female SV40-Tag mice, as depicted in Supplemental Figure 1. Mean values for 30 minute measurements during active and inactive periods +/- standard error of the mean are reported.

**Supplemental Table 4:** Metabolic cage measurements of isolated vs. grouped CD-1 mice. A summary table of the metabolic cage analysis for grouped and isolated female CD-1 mice, as depicted in supplemental figure 2. Mean values for 30 minute measurements during active and inactive periods +/- standard error of the mean are reported.

**Supplemental Figure 1**. Metabolic cage data for isolated vs. grouped SV40-Tag mice. At 15 and 18 weeks of age, three isolated and three group-housed mice were placed in individual metabolic cages. Results were analyzed for days six and seven, following five days of acclimation.

**Supplemental Figure 2**. Metabolic cage data plots for isolated vs. grouped CD-1 mice. At 15 weeks of age, three CD-1 mice from isolated and grouped housing were placed in individual metabolic cages. Results were analyzed for days six and seven, following five days of acclimation.